Ursolic acid induces cell death and modulates autophagy through JNK pathway in apoptosis-resistant colorectal cancer cells

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Abbreviations: CRC, colorectal carcinoma; 5-FU, 5-fluorouracil; UA, ursolic acid; MSI, microsatellite instability; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinase; NAC, N-Acetyl-L-cysteine; STS, staurosporine; TUNEL, TdT mediated dUTP Nick End Labelling; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Abstract

Colorectal carcinomas (CRC) with *P53* mutations have been shown to be resistant to chemotherapy with 5-fluorouracil (5-FU), the most widely used chemotherapeutic drug for CRC treatment. Autophagy is emerging as a promising therapeutic target for drug resistant tumors. In the present study, we tested the effects of ursolic acid (UA), a natural triterpenoid, on cell death mechanisms and its effects in combination with 5-FU in the HCT15 p53 mutant apoptosis resistant CRC cell line. The involvement of UA in autophagy and its *in vivo* efficacy were evaluated.

Our data shows that UA induces apoptosis independent of caspases in HCT15 cells, and enhances 5-FU effects associated with an activation of JNK. In this cell line, where this compound has a more pronounced effect on the induction of cell death compared to 5-FU, apoptosis corresponds only to a small percentage of the total cell death induced by UA. UA also modulated autophagy by inducing the accumulation of LC3 and p62 levels with involvement of JNK pathway, which indicates a contribution of autophagy on JNK-dependent induction of cell death by UA. By using nude mice xenografted with HCT15 cells, we verified that UA was also active *in vivo* decreasing tumor growth rate.

In conclusion, this study shows UA's anticancer potential both *in vitro* and *in vivo*. Induction of cell death and modulation of autophagy in CRC resistant cells was shown to involve JNK signalling.

Introduction

Colorectal carcinoma (CRC) is the second leading cause of cancer related death and 5-fluorouracil (5-FU) is the main chemotherapeutic agent used in the treatment of this disease [1]. However, significant resistance to 5-FU has been reported and other compounds are needed in order to increase treatment efficacy [2]. Resistance to 5-FU (with reduced induction of apoptosis) has been associated with tumour cells that harbour *P53* mutations [3-5]. Tumors presenting microsatellite instability (MSI) status, which accounts for 15% of sporadic CRC, have also demonstrated *in vitro* resistance to 5-FU [6-8], suggesting little or no benefit from 5-FU treatment in MSI patients, although clinical evidence is not always consistent [8]. These patients, in particular those with MSI and p53 mutations, would clearly gain from new treatment modalities for enhanced efficacy.

Apoptotic cell death is a fundamental cellular process that plays an important role during development and tissue homeostasis and has also a profound effect on cancer progression and response to treatment [9]. Apoptosis can be mediated by death receptors (extrinsic pathway) or by the mitochondrial pathway (intrinsic pathway), both involving the activation of caspases [9, 10]. Other alternative cell death mechanisms independent of caspases have been proposed, such as modulation of autophagy [11]. Autophagy is considered a mechanism of cell survival with an important role in preventing early phases of tumor development [12]. However, at late stages of tumor development it may confer anticancer drug resistance [13, 14]. Thus, inhibition of autophagy in resistant cancer cells can lead to cell death and it is currently considered an alternative therapeutic approach [13].

The c-Jun N-terminal kinase (JNK), a stress-activated protein kinase of the family of the mitogen activated protein kinase (MAPK), has been implicated in many

cellular events including apoptosis signalling [15, 16]. More recently, JNK was also found to be a mediator of autophagy, contributing to autophagic cell death in some types of cancer cells [17-22]. Activation of JNK can induce Beclin-1 expression [19], mediate damage-regulated autophagy modulator (DRAM) [18, 22], as well as, mediate p53 phosphorylation [17], effects that contribute to cell death.

Several phytochemicals have demonstrated the ability to modulate cancer cell death through different signalling pathways [23, 24]. Activities, such as anti-inflammatory and anticancer, have been attributed to ursolic acid (UA), a naturally occurring triterpenoid found in fruits and herbs [25]. In a previous study [26], we showed that UA has anticarcinogenic potential through inhibitory effects on PI3K pathway in HCT15 MSI mutant p53 CRC cell line. The present study demonstrates that UA induces cell death and modulates autophagy through JNK signaling. In addition, UA enhances 5-FU-induced apoptosis in this resistant cell line where it demonstrated to be even more efficient in inducing cell death than 5-FU alone. *In vivo* results using xenografted nude mice showed that UA significantly decreased tumor growth while increasing expression of autophagy markers (p62) and JNK, providing evidence for UA's therapeutic potential against CRC.

Material and methods

Reagents and antibodies

Ursolic acid (UA), z-VAD-fmk (zVAD), staurosporine (STS), 5-Fluorouracil (5-FU), SP600125 (SP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and N-Acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis,

MO, USA). UA, zVAD, STS, 5-FU and SP were used as stock solutions dissolved in dimethyl sulfoxide (DMSO).

Primary antibodies were purchased from the following sources: anti-phospho-JNK (G-7), anti-JNK, anti-p53 and anti-MAPLC3 (clone 5F10) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-phospho-mTOR and anti-mTOR from Cell Signaling (Danvers, MA, USA); anti-p62 (SQTM1) from Enzo Life Sciences (Lorrach, Germany); anti-LC3 (clone 5F10) from NanoTools (Teningen, Germany); and anti-β-actin from Sigma-Aldrich. Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchased from GE Healthcare (Bucks, UK).

Cell line and culture conditions

HCT15 and CO115 human colon carcinoma-derived cell lines were kindly provided by Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal). Cell lines were maintained at 37ºC in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10mM HEPES, 0.1mM pyruvate, 1% antibiotic/antimycotic solution (Sigma-Aldrich) and 10% fetal bovine serum (FBS; EU standard, Lonza, Verviers, Belgium). Cells were seeded onto six (2ml) and twelve (1ml) well plates at a density of 0.75×10^5 cells/ml. Test compounds were added to culture medium to the desired concentration ensuring that the DMSO concentration did not exceed 0.5% (v/v); controls received vehicle only.

Apoptosis analysis by TUNEL assay

TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed to estimate the percentage of cells with DNA damage typical of apoptosis. After the different treatments for 48h, cells were collected (both floating and attached cells), fixed with 4% paraformaldehyde for 15min at room temperature and attached onto a

polylysine treated slide using a Shandon Cytospin. Centrifuged cells were then washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2min on ice. TUNEL assay was performed using a kit from Roche (Mannheim, Germany), following the manufacturer's instructions. Cells were incubated with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of cells (nuclei staining with Hoechst), from a count higher than 500 cells per slide under a fluorescent microscope. Results are presented as mean ± SEM of at least three independent experiments.

Cell death analysis by PI staining

After the different treatments (2h or 48h), cells were collected (both floating and attached cells) and washed in ice cold PBS containing 5% (v/v) FBS. Cells were then resuspended in ice cold PBS with propidium iodide (PI) added to a final concentration of 0.5mg/ml. Cells were maintained on ice and protected from light. Twenty microliters of the stained cell suspensions were placed on clean microscope slides and overlaid carefully with coverslips. Immediately, cells were visualized on a fluorescent microscope and photos taken from different fields. The percentage of dead cells (PI positive) was calculated from the ratio between PI positive cells and total number of cells (visualized under phase contrast), from a count higher than 500 cells per slide. Results are presented as mean ± SEM of at least three independent experiments.

Western blot analysis

Cells were subjected to different treatment combinations for 24h or 48h, and total cell lysates were prepared to measure expression of different proteins. The cells were washed with PBS 1X and lysed for 15min at 4ºC with ice cold RIPA buffer (1%

NP-40 in 150mM NaCl, 50mM Tris (pH 7.5), 2mM EDTA), supplemented with 20mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 20mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA used as a protein standard. For western blot analysis, 20µg of protein were resolved by SDS-polyacrylamide gel and electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or 1% (w/v) BSA (bovine serum albumin), washed in TPBS and incubated with primary antibody overnight. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase for 1h and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-actin was used as loading control.

In vivo experiment of UA treatment in mice xenografted with HCT15 cells

Six to eight weeks-old female Balb/cA nude mice (Taconic BALBANU-F) were kept in individually filtered ventilated housing, and acclimated before the experiment. HCT15 cells (10^6 cells in 100µl Hanks BSS) were injected subcutaneously into the right flank of each animal and tumors were allowed to grow for one week. Mice were then assigned to two groups of ten animals: Group 1 placebo and Group 2 UA. Animals received orally once daily 0.1 ml of Nutella with or without UA (75mg/kg body weight) for 14 days. Tumor growth was measured twice a week for two weeks or until tumor volume was 1 cm^3 whichever was attained first. Tumor size was calculated using the

formula: $V = \frac{3}{4} \pi (a/2)^2 b$, where a represents the smallest tumor diameter and b the largest tumor diameter. No signs of toxicity were observed in animals. The experiment was carried out at the Biocenter, University of Copenhagen, according to the regulation of Danish national authorities for handling laboratory animals.

Histological sections and immunohistochemistry

At the end of the two weeks treatment period, tumors were excised, fixed in formalin and paraffin-embedded. Five µm sections were cut, collected onto APS coated slides, and dried at 37ºC overnight. For immunohistochemistry analysis, slides were deparaffinised, rehydrated and antigen retrieval was performed by placing slides in 0.05% citraconic anhydride solution, pH 7.3, for 30 min at 98ºC [27] and, after drying at 37ºC, 5 min incubation with 1% SDS in phosphate buffered saline (PBS). Sections were then blocked with 5% normal goat serum in 0.05% tween-20/1% bovine serum albumin/PBS and incubated with primary antibodies overnight at 4ºC in humidity chambers: rabbit ant-p62 (1:500), mouse anti-LC3 (5µg/ml) and mouse anti-p-JNK (1:100). After incubation, slides were washed with TPBS and incubated with secondary antibodies (goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568; Invitrogen) for 1 h at 37ºC. Slides were rinsed, nuclei were counter stained with DAPI and mounted with 10% Mowiol, 40% glycerol, 0.1% DABCO, 0.1 M Tris (pH 8.5). Slides were observed in a fluorescent microscope and semi-quantitatively scored, and photos taken in a confocal microscope.

Statistical analysis

Statistical analyses were done using *t*-test and two-away ANOVA, using GraphPad Prism 4.0 software (San Diego, CA, USA). *P*-values ≤ 0.05 were considered

statistically significant. All results are presented as mean ± SEM of at least 3 independent experiments. Images are representative of three independent experiments.

Results

UA induces caspase-independent apoptosis in HCT15 and enhances 5-FU effect

In a previous study, UA at 4μM was shown to decrease significantly cell proliferation (by 50%), to inhibit PI3K/Akt pathway and to induce apoptosis as assessed by TUNEL assay [26]. In the present study we aimed to clarify the mechanisms involved in the cell death induced by UA that is only partly due to apoptosis induction. As shown in Fig. 1A, the significant induction of TUNEL-positive cells by UA in HCT15 cells was caspase independent, since the inclusion of the inhibitor of caspases z-VAD (20μM) did not prevent the induction of apoptosis by UA after 48h of treatment. The increase of TUNEL-positive cells by the classical inducer of apoptosis staurosporine (STS, 0.250μM) was also independent of caspases in this apoptosis resistant cell line. These results were corroborated by analysis of apoptosis markers by western blotting (Fig. 1B), where UA did not induce the cleavage of caspase 9, caspase 3 or PARP-1. However, STS induced slightly the cleavage of PARP-1 and decreased the levels of procaspase 3 and 9 (Fig. 1B). These results suggest that UA induces apoptosis by a caspase-independent mechanism in HCT15 cells.

We had also shown previously that HCT15 cells are resistant to induction of cell death by apoptosis by a common CRC chemotherapeutic drug 5-FU, probably due to the p53 mutation and MSI status [28]. However, when we subjected HCT15 cells to the combination of UA with 5-FU for 48h, at concentrations that were previously shown to decrease cell growth by 50% [26, 28], a significant enhancement of apoptosis was

observed when compared with both compounds alone (Fig. 1C). Interestingly, this same combination of 5-FU and UA when tested in normal human fibroblasts did not decrease cell viability (Supplementary Fig. 1).

UA induces JNK-dependent apoptosis in HCT15

Since the stress-activated MAPK signalling pathways have been implicated in cell death mechanisms [16], we further studied their involvement in UA-induced apoptosis, as well as, the role of reactive oxygen species (ROS). As shown, in Fig. 2A, co-incubation of UA with the antioxidant N-acetylcysteine (NAC) inhibited the % of TUNEL-positive cells, suggesting an implication of oxidative stress as a contributor for UA-induced apoptosis in HCT15 cells. Previously, we observed that UA did not change MAPK/ERK pathway in HCT15 cells [26]. Here we studied the involvement of the stress kinases p38 and JNK on apoptosis induced by UA in HCT15 cells. Using western blot analysis, we observed that UA significantly induced phospho-JNK (active form) expression (Fig. 2B). An increase of phospho-JNK expression was also observed for STS and no effect was detected for 5-FU. UA did not change the expression of phospho-p38 expression in HCT15 cells (data not shown).

To assess whether apoptosis induction by UA and UA plus FU were dependent on JNK activation, incubations in the presence of 20μM SP600125 (SP), a JNK inhibitor, were performed. As shown in Fig. 2C, SP inhibited TUNEL-positive cells induced by UA, suggesting a dependence on JNK signalling for the UA-induced apoptosis in HCT15 cells. SP also inhibited TUNEL-positive cells induced by STS (Supplementary Fig. 2), an effect not observed with 5-FU (Fig. 2C). An almost complete abrogation of TUNEL-positive cells induced by the combination of UA with

5-FU was observed in the presence of SP (Fig. 2C). These results indicate that activation of JNK by UA is necessary for UA-induced apoptosis in HCT15 cells.

UA induces cell death and modulates autophagy through JNK pathway in HCT15

Although significant, apoptosis induced by UA in HCT15 only affects around 4% of total cell number, which does not reflect the extensive morphological changes (and appearance of floating cells) induced by this compound, suggesting a much higher percentage of cell death. Cell death was, therefore, subsequently measured using PI staining, at 2h and 48h. As shown in Supplementary Fig. 3, UA produced a small increase in cell death after 2h of incubation, indicating no acute necrotic effect. However, after 48h, UA induced cell death in around 50% of cells, as shown by the increase number of PI positive cells (Fig. 3A, Supplementary Fig. 3). On the other hand, 5-FU alone did not induce significant PI positive cell death and no cumulative effect with UA was observed (Fig. 3A). These results indicate that UA induces cell death in HCT15 cells more efficiently than 5-FU and also by mechanisms other than apoptosis. We also tested whether JNK pathway was involved in the total cell death induced by UA. As shown in Figure 3A, SP partially inhibited total cell death induced by UA, as well as, the cell death induced by the combination of UA with 5-FU. These data suggest a dependence on JNK signaling also for the total cell death induced by UA in HCT15 cells.

Recently, autophagy has been argued to be a potential target for induction of cell death in chemoresistant cancer cells [13]. Therefore, we further investigated the possible role of UA in autophagy and the involvement of JNK in this process. As shown in Fig. 3B, UA induced an accumulation of both LC3-II (and to a lower extent also LC3-I) and p62 levels in HCT15 cells after 48h of treatment, which were remarkably

prevented in the presence of SP, indicating a role of JNK activation in the accumulation of these autophagic mediators. No effect on LC3-II protein expression was detected in cells treated with 5-FU or SP alone (Fig. 3B). The LC3-II accumulated in cells treated with UA plus 5-FU seems to be due to UA alone, an effect also inhibited in the presence of SP. The possible role of UA in some upstream regulators of autophagy, such as p53 and mTOR, was also investigated. Figure 3B shows that UA decreased both p53 and phospho-mTOR levels, as well as, the levels of p53 induced by 5-FU. These effects are consistent with the potential of UA to modulate autophagy.

Interestingly, UA also modulates the same autophagic mediators in other CRC cells, such as the MSI CO115 p53 wild-type cell line (Supplementary Fig. 4D). Although in this cell line UA induced apoptosis dependent of caspases, total cell death is higher (Supplementary Fig. 4A-C) indicating a possible role of autophagy on cell death.

UA decreases tumor growth in mice xenografted with HCT15 cells

Because UA demonstrated to induce cell death more efficiently than 5-FU in HCT15 cells, we evaluated *in vivo*, in mice xenografted with HCT15 cells, the effects of UA and the possible implication of autophagy and JNK signaling on tumor growth. As shown in Fig. 4A, UA significantly decreased tumor growth rate after 14 days of treatment when compared to the control group. No significant effect on body weight was observed between treatments (data not shown). Using immunohistochemistry analysis of these tumors, a tendency for a higher expression of p62 and phospho-JNK in UA group was observed as compared to controls (Fig. 4B, 4C). Colocalization of p62 and phospho-JNK was, however, only partial. No differences were observed on the tumor expression of LC3 or the proliferation marker Ki67 between treatments (Fig. 4B,

4C). These results suggest the potential of UA in reducing tumor growth and the possible involvement of JNK signalling and autophagy in the *in vivo* effects of UA.

Discussion

Several mechanisms of resistance have been reported that decrease 5-FU efficacy, such as loss of p53 transactivation function [3-5]. Defects in apoptosis play a central role in tumorigenesis and confer resistance to anticancer therapies [29]. Alternative strategies such as autophagy inhibition have been demonstrated to sensitize tumor cells to anticancer drugs [13, 14]. In a previous study, we showed that UA induces apoptosis in HCT15 mutant p53 MSI human CRC cell line [26]. This cell line is also resistant to 5-FU [28], and here we showed that combination of this chemotherapeutic agent with UA significantly enhanced apoptosis as compared with 5-FU alone. This drug combination did not have any cytotoxicity in normal cells. The induction of apoptosis in HCT15 cells by UA (with or without 5-FU) was shown to be dependent on JNK pathway and independent of caspases. In the conditions used, the apoptosis induced by UA is small and did not account for the total cell death (about 50%) observed, suggesting that other mechanisms must be involved.

Reactive oxygen species (ROS) have been shown to be involved in the regulation of cell death and signalling through JNK pathway [30]. This pathway is implicated in many cellular events related to cell death, such as apoptosis [15, 16] and autophagy [17-22]. Our results showed that the antioxidant NAC partially inhibited apoptosis induced by UA, suggesting the involvement of ROS on UA´s effects. In addition, UA activated JNK pathway, as shown by the increased levels of phospho-JNK. Its inhibition with SP significantly decreased UA-induced cell death and the increase of the autophagic mediators LC3 and p62. Therefore, both apoptosis and total

296 cell death induced by UA alone or UA in combination with 5-FU were shown to involve
297 JNK pathway, possibly in response to oxidative stress produced by UA. The importance
298 of JNK activation as one contributing mechanism to cell death induction in CRC has
299 previously been demonstrated for atorvastatin. This drug was shown to induce apoptosis
300 involving JNK activation and to synergistically interact with celecoxib, a selective
301 cyclooxygenase-2 inhibitor, in killing human CRC cells [31]. Also, UA has been shown
302 to induce JNK pathway in other cell lines [32-37] leading to cell death, however its
303 association with autophagy has never been reported.

304 Autophagy is activated under stress conditions, such as nutrient and/or growth
305 factor deprivation and, although it represents a mechanism of survival, it may assume a
306 cell death function in cancer cells when apoptosis is deregulated [11, 12]. Several
307 signalling proteins have been demonstrated to interfere with autophagy [13, 38]. In our
308 previous work, UA showed to decrease PI3K/Akt pathway [26]. Here, we observed that
309 UA also decreased the levels of phospho-mTOR, as well as, the levels of mutant p53.
310 Since an inhibition of mTOR is associated with an induction of autophagy [38, 39] and
311 the cytosolic mutant p53 has shown to inhibit autophagy [40, 41], our results suggest
312 that UA may induce autophagy by inhibiting the PI3K/Akt/mTOR signaling and
313 decreasing mutant p53.

314 On the other hand, the ability of UA to increase the levels of autophagic
315 mediators LC3 and p62 suggest that UA may be inhibiting autophagy. LC3-II is
316 associated with autophagosome membrane reflecting its abundance. Its increasing levels
317 have been interpreted as either the result of induction or inhibition of the autophagic
318 process [42]. However, the accumulation of both LC3-I and LC3-II after long periods of
319 incubation, as observed here, is taken as an indication of inhibition of autophagy [42].
320 In the case of p62 that is selectively incorporated into autophagosomes through binding

to LC3, its levels reflect its degradation by autophagy [42]. Therefore, an accumulation of p62 represents an inhibition at later steps of the autophagic process. As a result, in HCT15 cells, modulation of autophagy by UA seems to involve a dual effect: it may facilitate initial stages but it inhibits autophagy at later steps. Nevertheless, UA seems to be able to modulate autophagy independently of p53 status and of apoptosis cell resistance, since UA had the same effects in other CRC cells, such as CO115 cell line. Further experiments have to be performed to elucidate in detail the effects of UA on autophagy and its implication on induction of cell death.

Since UA alone was more efficient in inducing cell death than 5-FU *in vitro*, the *in vivo* potential of UA against CRC was evaluated in nude mice xenografted with HCT15 cells. Interestingly, UA decreased tumor growth rate after 2 weeks of treatment, without affecting body weight, and a tendency to increase the levels of p62 and phospho-JNK in tumors was observed. These results suggest that the antitumor effect of UA may involve the regulation of autophagy possibly by JNK signaling. The *in vivo* potential of UA as an antitumorogenic agent has recently been suggested in other cancer types [43, 44].

In conclusion, this study shows that UA enhances the apoptotic effect of 5-FU, with an activation of JNK. UA induces cell death in CRC resistant cell line more efficiently than 5-FU probably by inhibiting autophagy. The antitumor potential of UA against CRC and the possible involvement of autophagy and JNK were observed *in vivo*. The applicability of UA as a potential inhibitor of autophagy should be explored in future studies and in strategies for treatment of CRC tumors resistant to conventional chemotherapeutic drugs.

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Figure Legends

Figure 1 – Effect of both ursolic acid (UA) and 5-fluorouracil (FU), alone or in combination, on apoptosis in HCT15 cells. (**A**) Effect of UA 4µM (UA4) and staurosporine (STS) 0.25µM with or without a caspase inhibitor zVAD-FMK (zVAD) 20µM in the % of TUNEL-positive cells after 48h of treatment. (**B**) Effect of UA4 for 24h on the expression of protein markers of caspase-dependent apoptosis, as assessed by western blotting. (**C**) Effect of UA4 alone or in combination with 5-fluorouracil 100µM (FU100) for 48h in the % of TUNEL-positive cells. **(A, C)** Values are mean ± SEM of at least 3 independent experiments. * P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001, when compared with control (CT); ++ P≤ 0.01, when compared with the respective compound alone; ## P≤ 0.01 and ### P≤ 0.001, when compared with FU or zVAD alone; NS, not significant differences observed between each other. In **B**, images are representative of at least 3 independent experiments with similar results. β-actin was used as loading control.

Figure 2 – Effect of JNK pathway on apoptosis induced by ursolic acid (UA) in HCT15 cells. (**A**) Effect of N-Acetyl-L-cysteine (NAC) 5mM in the % of TUNEL-positive cells induced by UA 4µM (UA4) after 48h of treatment. Values are mean ± SEM of at least 3 independent experiments. *** P≤ 0.001, when compared to control (CT); ### P≤ 0.001, when compared with NAC alone; ++ P≤ 0.01, when compared with each other. (**B**) Effect of UA4, staurosporine (STS) 0.25 µM and 5-fluorouracil 100µM (FU100) on phospho-JNK and total JNK levels, for 48h, using western blot. Images are representative of at least 3 independent experiments with similar results. β-actin was used as loading control. (**C**) Effect of UA 4µM, FU 100µM and SP600125 (SP) 20µM, a JNK inhibitor, alone or in combination, in the % of TUNEL-positive cells for 48h of treatment. Values are mean ± SEM of at least 3 independent experiments. ** P≤ 0.01, *** P≤ 0.001, when compared with UA alone; θ P≤ 0.05 and θθ P≤ 0.01, when compared with SP alone; ### P≤ 0.001, when compared with FU alone. ++ P≤ 0.01 and +++ P≤ 0.001, when compared with each other; NS, not significant when compared with each other.

Figure 3 – Effect of both ursolic acid (UA) and 5-fluorouracil (FU), alone or in combination, on cell death and autophagic mediators in HCT15 cells. (**A**) Effect on cell death of UA 4µM, FU 100µM and SP600125 (SP) 20µM, alone or in combination, for 48h, as assessed by PI staining. Values are mean ± SEM of at least 3 independent experiments. *** P≤ 0.001, when compared with UA alone; θθθ P≤ 0.001, when compared with SP alone; ### P≤ 0.001, when compared with FU alone; ++ P≤ 0.01 and +++ P≤ 0.001, when compared with each other; NS, not significant when compared with each other. (**B**) Effect UA 4µM, FU 100µM and SP 20µM, alone or in combination, in the levels of LC3, p62, p53, phospho-mTOR and total mTOR, for 48h, using western blot. Images are representative of at least 3 independent experiments with similar results. β-actin was used as loading control.

Figure 4 – Effect of ursolic acid (UA) treatment *in vivo*. (**A**) Tumor progression of HCT15 cells xenografted in nude mice for 14 days, as expressed by relative tumor size. Mice were divided in two groups: control (placebo) and UA (75 mg/kg), each with 7 animals. The effect of time ($p < 0.001$) and treatment were observed ($p < 0.001$), as well as, the effect of the interaction ($p=0.006$) shown in the figure as (++). (**B**) Representative confocal images of immunohistochemical analysis of the expression of p62 (green), ki67 (green), phospho-JNK (red) and LC3 (red) proteins in the tumors treated with UA in the *in vivo* experiment; bar: 20µm. (C) Semi-quantification of the immunohistochemical sections of the expression of p62, phospho-JNK, LC3 and Ki67 proteins *in vivo*. Immunoreaction intensity was scored as 0 for negative staining, 1 for weak, 2 for intermediate and 3 for strong. Values are mean ± SEM of 7 animals each.

Figure 1

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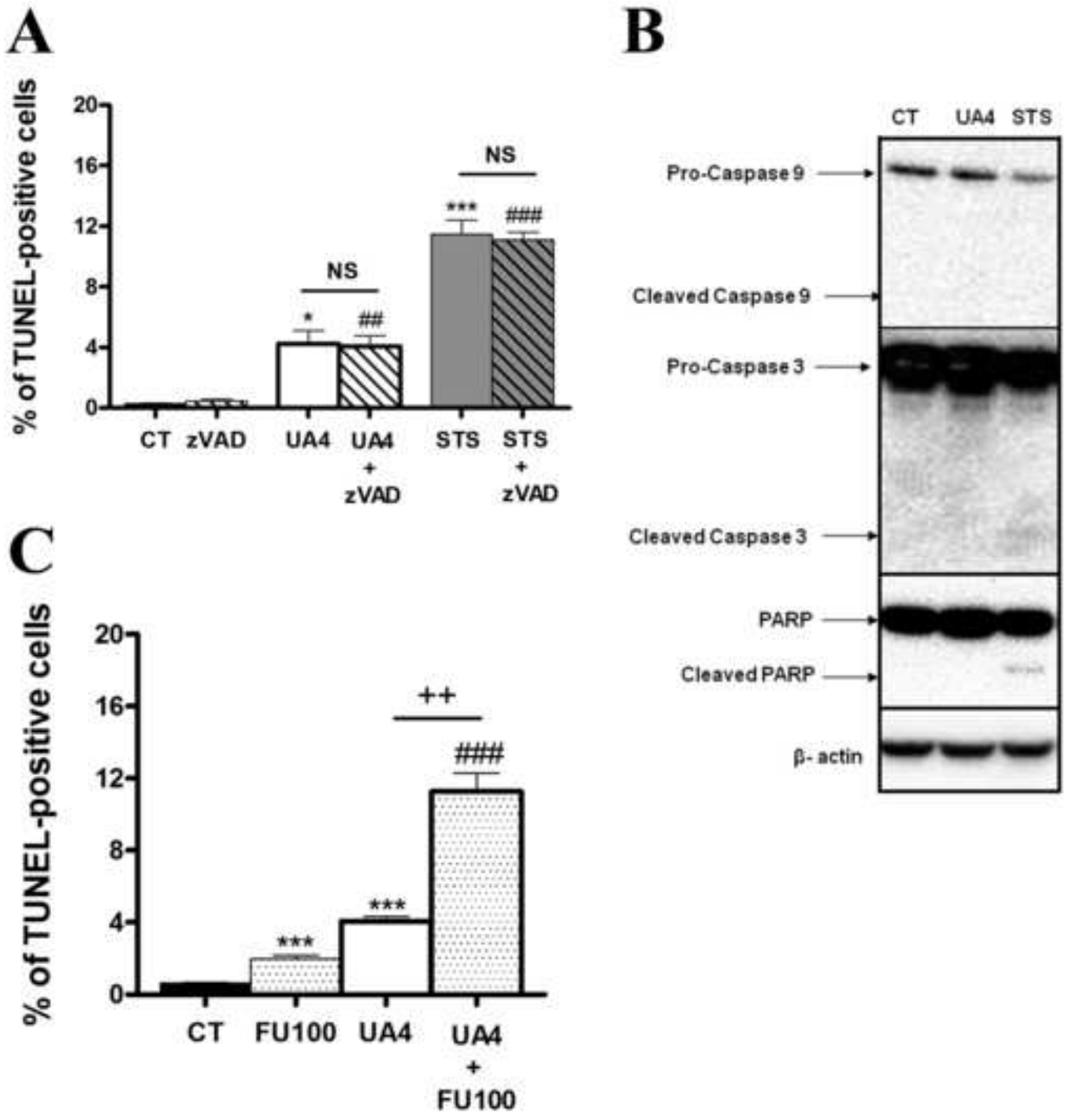

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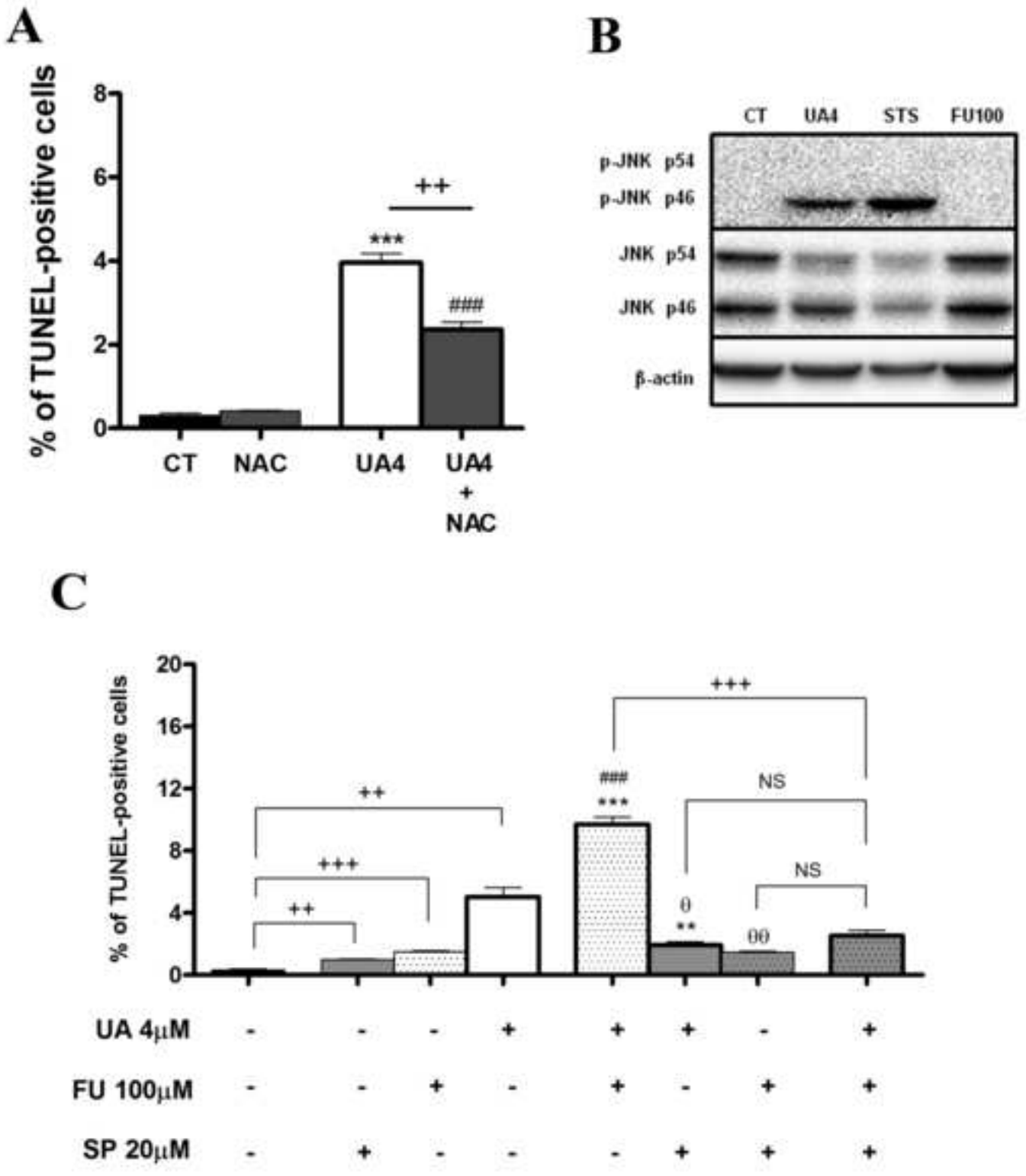

Figure 3

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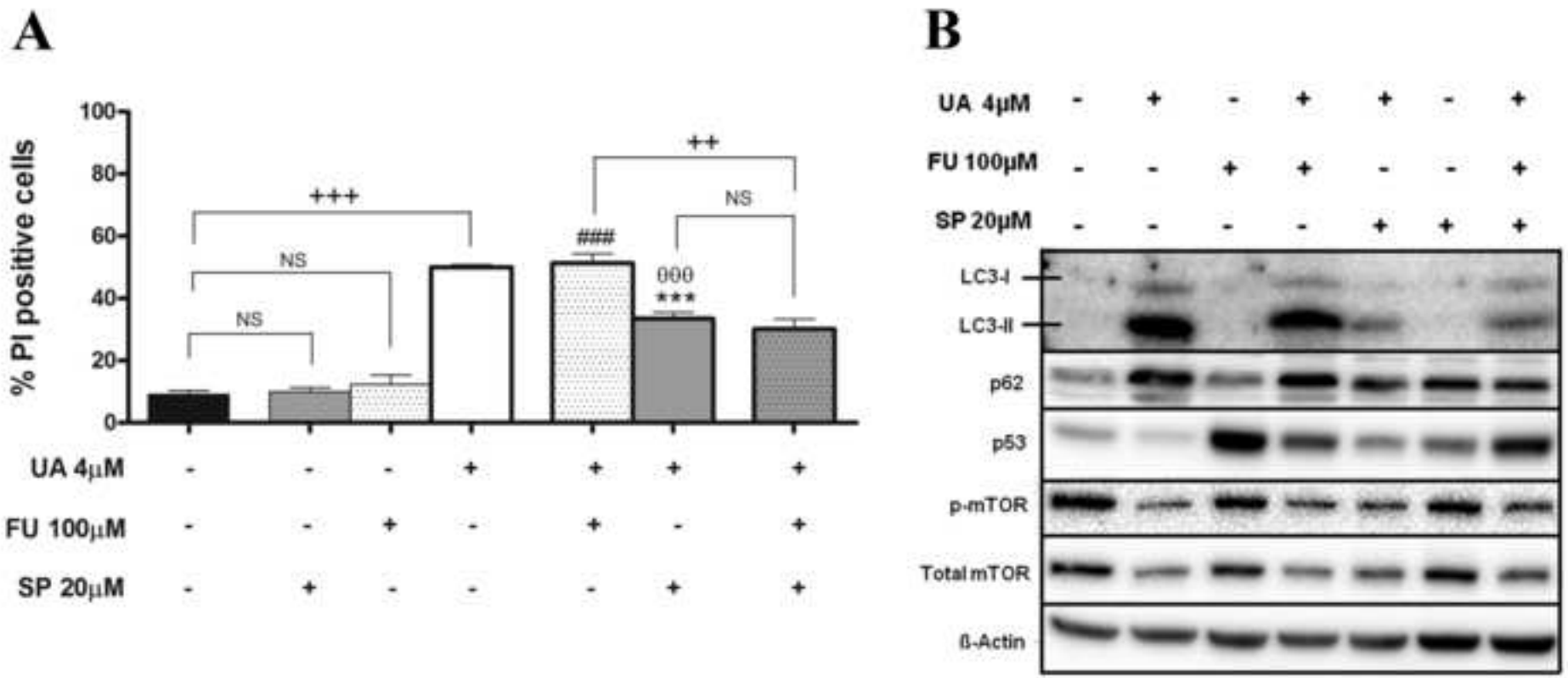

Figure 4

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